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Procedia Environmental Sciences 15 (2012) 56 – 65

Procedia Environmental Sciences

The 1st Asia Oceania Algae Innovation Summit (AOAIS-1)

Biosynthesis of isoprene units in the C_{34} botryococcene molecule produced by *Botryococcus braunii* strain Bot-22

Atsushi Ishimatsu[a], Hiroshi Matsuura[a], Tomoharu Sano[b] Kunimitsu Kaya[a], Makoto M. Watanabe[a]*

[a]*Graduate school of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennoudai Tsukuba, Japan*
[b]*National Institute for Environmental Studies, 16-2 Onogawa Tsukuba, Japan*

Abstract

The colonial green alga *Botryococcus braunii* produces a large amount of hydrocarbons. This alga is subclassified into three chemical races (A/B/L), according to the hydrocarbon structures. Strain Bot-22 isolated from a Japanese dam is classified as race B. The main product of the strain was C_{34} botryococcene which was determined by nuclear magnetic resonance (NMR). The results of [1-^{13}C] glucose feeding and NMR experiments showed that the compound was synthesized via the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway. These results roughly agreed with a previous study. Unexpectedly, ^{13}C-labeled methyl groups were detected in the ^{13}C-incorporated compound suggesting that pathways besides MEP are playing a role in biosynthesis.

Keywords: Botryococcus braunii; isoprenoid, botryococcene

1. Introduction

The colonial green alga *Botryococcus braunii* produces large amounts of lipids containing many kinds of hydrocarbons, triglycerides, and pigments. It is considered as a promising biolium producer [1]. Lipids are mainly located at two sites in the cell; the aliphatic outer cell wall (socket wall) and the intracellular oil body [2] [3] and different lipids accumulate at each site [4]. *B. braunii* is subclassified into three chemical races (A/B/L), according to the hydrocarbon structures: odd-carbon-numbered n-alkadienes

* Corresponding author. Tel.: +81-29-853-4301; fax: +81-29-853-4301
E-mail address: makoto@sakura.cc.tsukuba.ac.jp

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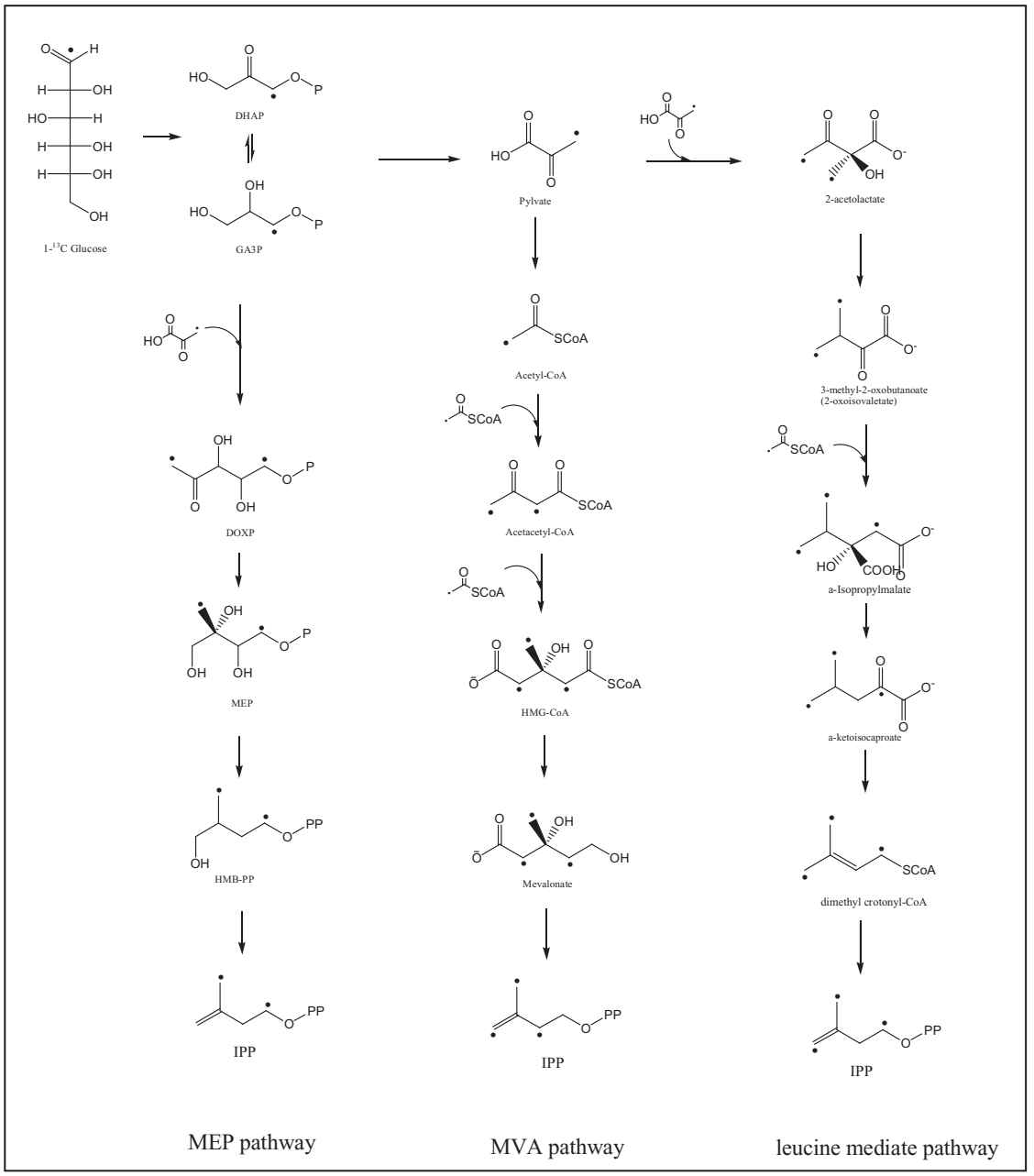
doi:10.1016/j.proenv.2012.05.010

and/or -trienes and their derivatives are synthesized by race A. Specific C_nH_{2n-10} triterpenes (n = 30–37), called botryococcenes, are synthesized by race B, and a tetraterpene called lycopadiene is synthesized by race L.

Isoprenoids including triterpenoids are detected in most living organisms and are involved in many cell processes as pigments, hormones, and membrane lipids. More than 30,000 isoprenoids have been identified. Isoprenoids consist of branched C5 isoprenic units derived from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Repetition of the linkage reaction converts IPP and DMAPP into many kinds of isoprenoids. Common precursors of isoprenoids are synthesized from some putative precursors i.e., mevalonate, deoxyxylulose [5], some amino acids [6], and pentose phosphate cycle substrates in *Synechocystis* [7]. The biosynthetic route from mevalonate is well known as the mevalonate (MVA) pathway which is preserved in many organisms, including animals, fungi, bacteria, and plant cell cytoplasm. The MVA pathway begins with three acetyl-CoA molecules. The rate determining step is the reduction of 3-hydroxy-3-methylglutaryl-CoA to generate mevalonate. The route from deoxyxylulose is called the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway and is associated with the intermediates. This pathway has been found in some bacteria including cyanobacteria and chloroplasts [8] [9]. Previous work has suggested that this is the sole pathway for isoprenoids in green algae such as *Scenedesmus obliquus* [10], *Chlamydomonas reinhardtii*, and *Chlorella fusca* [11]. Glyceraldehyde 3-phosphate and pyruvate are starting materials for the MEP pathway. On the pathway related to amino acids, the L-leucine skeleton is converted to IPP and DMAPP without the breakdown to acetyl-CoA. There are common reaction steps to the MVA pathway, however the isoprenic units are derived from two molecules of pyruvate and one molecule of acetyl-CoA [12]. The biosynthetic processes of the pentose phosphate cycle substrates are not known in detail. However, it is known that pentose phosphate cycle substrates are converted to downstream intermediates of the MEP pathway.

^{13}C-labelled positions on the products was convenient, as the isoprenic units are converted to products without reformation of the carbon skeleton. A previous report suggested that the MEP pathway is mainly used to produce botryococcenes and methylated squalene in the Berkeley strain. [1-^{13}C] glucose-incorporated positions derived from the MEP pathway show about an 80% higher isotopic abundance than other strains [15]. Moreover, a ^{14}C substrate-feeding experiment showed that only a small amount of mevalonate was incorporated into botryococcene [16]. Thus, the MVA pathway was considered not to be associated with botryococcene synthesis. In the course of ^{13}C-labelling studies on the biosynthetic route of isoprenic units in a new strain of *B. braunii* race B, Bot-22, unexpected ^{13}C-labelled methyl groups were detected in the ^{13}C-incorporated compound, suggesting the possibility that pathways besides MEP are involved in botryococcene biosynthesis. In this study, we show the details of the ^{13}C-labelling experiments and discuss the origin of the methyl group donor.

Since *B. braunii* race B accumulates a large amount of triterpenoids, up to 86% of dried cell weight [13], the biosynthetic system is required to generate IPP and DMAPP. ^{13}C-labeled substrates have usually been used in research to survey the origin of isoprenic units [14]. A labeled substrate is incorporated into different positions of the isoprenic units in response to each biosynthetic route (Scheme 1). Analysis of ^{13}C-labeled positions on the products was convenient, as the isoprenic units are converted to products without reformation of the carbon skeleton. A previous report suggested that the MEP pathway is mainly used to produce botryococcenes and methylated squalene in the Berkeley strain. [1-^{13}C] glucose-incorporated positions derived from the MEP pathway show about an 80% higher isotopic abundance than other strains [15]. Moreover, a ^{14}C substrate-feeding experiment showed that only a small amount of mevalonate was incorporated into botryococcene [16]. Thus, the MVA pathway was considered not to be



Scheme 1: Biosynthesis routes of isoprenic units. Close circles show positions with ^{13}C isotope label by 1-^{13}C glucose. Different label patterns were recorded according to mentioned three pathways.

associated with botryococcene synthesis. In the course of ^{13}C-labelling studies on the biosynthetic route of isoprenic units in a new strain of *B. braunii* race B, Bot-22, unexpected ^{13}C-labelled methyl groups were detected in the ^{13}C-incorporated compound, suggesting the possibility that pathways besides MEP are

involved in botryococcene biosynthesis. In this study, we show the details of the ^{13}C-labelling experiments and discuss the origin of the methyl group donor.

2. Method

2.1 Algal strain and culture condition

B. braunii Bot-22 strain, isolated by Dr. Kawachi, National Institute for Environmental Studies, Japan from a reservoir (Okinawa prefecture, Japan) was used. This strain has been maintained in our laboratory by subculturing using a screw-cap tube (18 mm Φ× 150 mm, Fujimoto Rika, Tokyo, Japan) with 10 mL of modified AF6 medium [17]. AF6 medium with glucose (10 mM) was used for the preculture in the labeling experiment, as the Bot-22 strain can take up glucose or other sugars as a carbon source.

Cells were grown in 1-L conical flasks with 500 ml of AF6 medium, which was aerated using sterile air at a flow rate of 100 mL/min, for hydrocarbon analysis. The cells were harvested in the stationary phase using GF/C filter paper (Whatman International Ltd, Maidstone, UK) , freeze-dried, and stored below -20°C.

For the ^{13}C-labeling experiment, cells were grown in a 200 mL flask with 100 mL of AF6 medium and 10 mM [1-^{13}C] glucose for 30 days. The culture was aerated using CO_2-omitted air which was prepared by bubbling air into a solvent saturated with NaOH and 95% H_2SO_4. All experiments were conducted at 25°C under a 12L-12D photoperiod cycle with a light irradiance of 100 μ mol photon/m^2 s.

2.2 Extraction and purification of hydrocarbons

Lipids were extracted from a wet biomass harvested at room temperature using 2:1 $CHCl_3$/MeOH (v/v). The extracts were concentrated under reduced pressure, and non-lipid materials were removed by adding one-fifth volume of 0.9% NaCl solution (w/w). After salting out, the $CHCl_3$ fraction was concentrated. The $CHCl_3$ solution was applied to a silica gel column prepared with $CHCl_3$, and four bed volumes of $CHCl_3$ were applied to the column. After evaporating the eluate, the remaining residue was dissolved with n-hexane. The hexane solution was applied to a silica gel column prepared with n-hexane, and then four bed volumes of n-hexane were applied to the column. The eluate was used for botryococcene analysis. A homolog of botryococcene was analyzed using a gas chromatography system (GC-2010, Shimadzu, Kyoto, Japan) equipped with a DB-5MS column which was programmed to heat from 130 to 270°C (20°C/min) and then from 270 to 300°C (4°C/min). The flame ionization detector and sample injector were programmed to maintain 320°C. The gas entered the column at 49 cm/s. High resolution GC-electron impact mass spectrometry (EIMS) analysis was also performed to determine the molecular weight of the main compound.

2.3 Identification of main component of hydrocarbon from BOT-22 strain

^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy including information from distortionless enhancement by polarization transfer (DEPT), H-H correlation spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC), and heteronuclear multiple bond correlation (HMBC) were performed to identify the main compound. Assignment of ^{13}C-labelled botryococcene was performed by matching with the ^{13}C-NMR spectra of non-labelled botryococcene produced by this strain. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded using a JEOL JNM-ECA500 spectrometer (JEOL ltd, Tokyo, Japan). $CHCl_3$ was used as the solvent and internal standard (δ_H 7.26 or δ_c 77.0). ^{13}C conversion was evaluated by comparing the peak heights from ^{13}C NMR. However, as it was difficult to

compare the peak height of the ^{13}C NMR directly because of signal enhancement by H-C decoupling and a concentration difference, a relative value was established as the division of signal heights from the ^{13}C feeding and control (value = feeding/control). Although this value has no significance for a quantitative analysis, it reflects the relative abundance of ^{13}C label between peaks.

Table 1. NMR spectra of main component of C34 botryococcene.

Position	H	C	DEPT	HMBC(C→H)
1	4.7 (brs)	[a] 109.5	CH2	3 27
2		[b] 150.0	C	3 28
3	2.17 (m)	[c] 41.1	CH	1 4 5 27 28
4	1.45 (m)	33.5	CH2	3 5
5	1.9 (m)	[d] 31.8	CH2	3 4 23
6		155.0	C	5 23 29
7	2.05 (m)	40.2	CH	23 29
8	1.38 (m)	30.2	CH2	9 29
9	1.31 (m)	39.2	CH2	11 30
10		41.9	C	9 11 12 24 25 30
11	5.3 (d 15)	135.9	CH	9 12 25 30
12	5.15 (dd 15,8)	133.8	CH	11 14 31
13	2.02 (m)	37.3	CH	11 12 14 31
14	1.25 (m)	35.1	CH2	31
15	1.45 (m)	33.6	CH2	32
16	2.01 (m)	40.7	CH	26 32
17		154.8	C	18 26 32
18	1.9 (m)	[d] 31.8	CH2	19 20 26
19	1.45 (m)	33.5	CH2	18 20 33
20	2.17 (m)	[c] 41.1	CH	18 19 22 33 34
21		[b] 150.0	C	20 33 34
22	4.7 (brs)	[a] 109.5	CH2	20 34
23	4.7 (brs)	107.4	CH2	
24	4.94 (dd 10,2)	111.0	CH2	
	4.91 (dd 17,2)			
25	5.77 (dd 17,11)	147.0	CH	9 11 24 30
26	4.7 (brs)	107.3	CH2	
27	1.67 (s)	19.0	CH3	1 3
28	1.03 (d 7)	19.8	CH3	3
29	0.99 (d 7)	20.3	CH3	8
30	1.04 (s)	23.7	CH3	9 11 25
31	0.96 (d 7)	21.2	CH3	14
32	0.99 (d 7)	20.4	CH3	
33	1.03 (d 7)	19.8	CH3	20
34	1.67 (s)	19.0	CH3	20

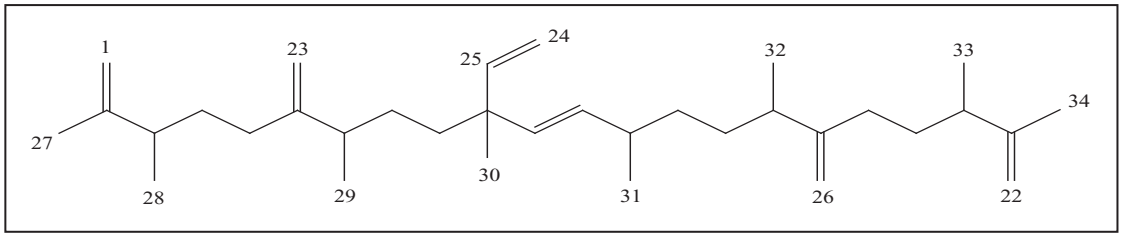


Fig. 1. Structure of the main hydrocarbon.

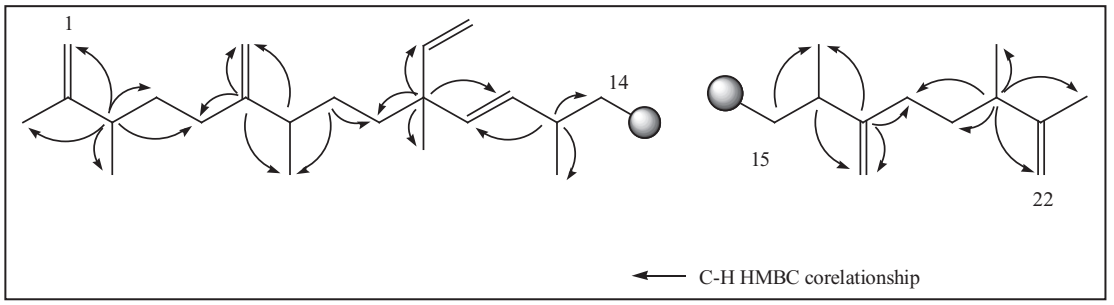


Fig. 2. Two large partial structures supported by HMBC.

3. Result and discussion

3.1 Identification of main component of hydrocarbon from Bot-22 strain

Further isolation was not performed, as the preliminary GC analysis suggested that the hexane elution contained a botryococcene with a sufficiently high purity (>95%) to be analyzed by NMR. In the high resolution EIMS analysis, the main botryococcene was observed at m/z. 466.4543 [M^+] (calculated for $C_{34}H_{58}$, D + 0.4 mmu).

The NMR spectra results are shown in Table 1. The main hydrocarbon of strain Bot-22 was identified as shown in Figure 1. The structure was the same as that of C_{34} botryococcene [18]. In the ^{13}C NMR spectrum, four signals at 109.5, 150.0, 41.1, and 31.8 ppm completely overlapped, indicating that the structure contained symmetric moieties.

The chemical formula suggests that the molecule has six double bonds. Twelve double bond carbons were confirmed by the NMR spectrum (δ_C > 100 ppm). The NMR spectra with DEPT and HMQC provided information about the carbon classes. Five unsaturated methylene carbons were found at 109.5, 109.5, 107.4, 111.0, and 107.3 ppm [C-1, 22, 23, 24, 26]. Apparently, one consisted of a vinyl group, as the coupling constants between H-24 (δ_H 4.94, 4.91) and H-25 (δ_H 5.77) were 17 Hz and 10 Hz each, and additionally that of H-24 was 2 Hz. Moreover, a double bond in the main chain existed at H-11 (δ_H 5.3 d) and H-12 (δ_H 5.15 d). The coupling constant between H-11 and H-12 (15 Hz) suggested a trans double bond linked to a quaternary and tertiary carbon. These couplings were confirmed by H-H COSY spectra. For coupling of the ^1H NMR [H-1, 22, 23, 26] singlet, all other saturated methylenes were double bonded with quaternary carbons [C-2, 6, 17, 21]. A quaternary carbon without a double or triple bond was also detected in C-10 (δ_C 41.8). Eight primary carbons [C-27, 28, 29, 30, 31, 32, 33, 34] indicated a near chemical shift on ^{13}C NMR; however, the ^1H NMR spectra of H-27 and H-34 (δ_H 1.67 s) clearly showed

Table 2, the signal height of ^{13}C NMR spectra by [1-^{13}C] glucose feeding and control.

Position	Chemical shift	peak height (%)		value	Position	chemical shift	peak height (%)		value
		feed(a)	control (b)	(a/b)			feed(a)	control (b)	(a/b)
1	109.5	100.0	11.9	8.4	**18**	31.8	8.1	13.1	0.6
2	150.0	2.9	3.7	0.8	**19**	33.5	69.6	7	9.9
3	41.1	4.1	11.5	0.4	**20**	41.1	4.1	11.5	0.4
4	33.5	40.7	9.1	4.5	**21**	150.0	2.9	3.7	0.8
5	31.8	8.1	13.1	0.6	**22**	109.5	100.0	11.9	8.4
6	155.0	2.4	2.1	1.1	**23**	107.4	44.1	6.7	6.6
7	40.2	3.0	5.6	0.5	**24**	111.0	37.9	5.9	6.4
8	30.2	41.7	7.1	5.9	**25**	147.0	3.0	5	0.6
9	39.2	4.8	7	0.7	**26**	107.3	41.0	5.7	7.2
10	41.9	3.2	4	0.8	**27**	19.0	7.2	6.4	1.1
11	135.9	35.3	5.3	6.7	**28**	19.8	59.2	6.4	9.2
12	133.8	2.7	5.4	0.5	**29**	20.3	54.9	6.5	8.4
13	37.3	5.8	6.5	0.9	**30**	23.7	46.1	6.9	6.7
14	35.1	4.6	6.3	0.7	**31**	21.2	47.3	6.7	7.1
15	33.6	69.6	7.3	9.5	**32**	20.4	54.3	6.7	8.1
16	40.7	3.2	6.5	0.5	**33**	19.8	54.3	8.3	6.5
17	154.8	2.2	2.8	0.8	**34**	19.0	7.8	6.7	1.2

that C-27 and C-34 were linked to quaternary carbons with double bonds. Moreover, C-30 (δ_H 1.04 s) was clearly linked to C-10 as a quaternary carbon without a double bond. Others [C-28, 29, 31, 32, 33] showing ^1H NMR coupling as doublet were linked to tertiary carbons without double bonds. The HMBC spectra allowed conversion of those carbons into two large moieties, as shown in Fig. 2. Several pairs of H-C couplings could not be separated due to signal overlaps. Nevertheless, it did not influence the identification results, as C-3 to H-22 duplicated to H-1. Finally, two parts were connected at C-14 and C-15 as the only two remaining nodes; hence, the main component was identified as C_{34} botryococcene.

3.2 Biosynthesis pathway of isoprenic units for C_{34} botryococcene

The 30 day feeding experiment using [1-^{13}C] glucose with removal of CO_2 allowed the product to achieve quite high signals of ^{13}CNMR. It is possible that $^{13}CO_2$ generated from [1-^{13}C] glucose is re-incorporated, disturbing the label pattern. However, the result shows that the ratio of recycle ^{13}C carbon was not so high to disturb clear labelling. The results of the NMR spectra are shown in Table 2. A series of carbons influenced the amplitude strength by H-C decoupling. Additionally, signal overlaps also increased its amplitude. The ^{13}C signal intensities of several carbons in the molecule increased when ^{13}C was incorporated into botryococcene. The relative signal intensities were evaluated using a calculation (feeding/control on signal height). The results showed that, [C-1, 4, 8, 11, 15, 19, 22, 23, 24, 26, 28, 29, 30, 31, 32, 33] were labelled remarkably with ^{13}C, and the intensity was 4.5–9.9 times higher than those of the control. The labelling pattern of C_{34} botryococcene is shown in Fig. 3a. The labelling patterns predicted from the three well-known pathways MEP, MVA, and leucine-mediated, are shown in Figs. 4b,

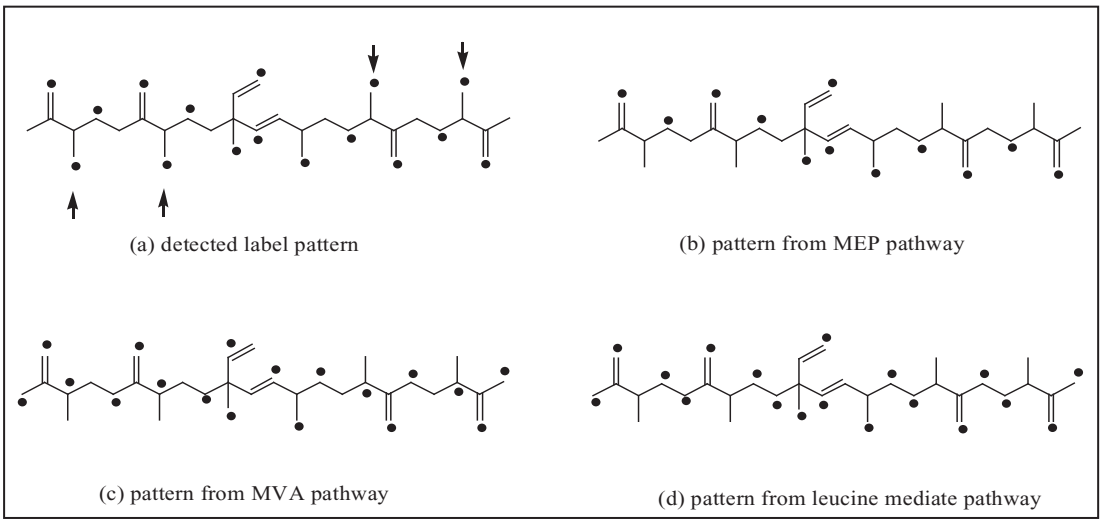


Fig. 3. Comparison of label pattern. (a) Detected label pattern by [1-^{13}C] feeding experiment. Arrows show the position of additional methyl base. (b)Label pattern predicted from the MEP pathway, C-1 and C-5 carbon on each isoprenic units were labelled. (c)Label pattern predicted from the MVA pathway. (d)Label pattern predicted from leucine mediate pathway.

c, and d. The labelling pattern completely coincided with the pattern that occurred when the MEP pathway was used to biosynthesize the isoprenic units. In contrast, the positions when the other two pathways were used showed fairly low signal peaks. We confirmed that the MEP pathway was used mainly for botryococcene biosynthesis in Bot-22 strain as well as the Berkeley strain.

It is unknown whether pentose phosphate cycle substrates participate in the biosynthesis of C_{34} botryococcene because this biosynthetic route has not been sufficiently investigated to anticipate the same labeling pattern from the reaction process. Further study will be needed to reveal the details of this pathway.

In this study, the ^{13}C from [1-^{13}C] glucose was detected at [C-28, 29, 32, 33] (Fig. 3a, indicated by the arrows). These carbons did not consist of any isoprenic units. The additional methyl groups were linked with the second carbons of the isoprenic units. These findings suggest that all botryococcenes were synthesized from C_{30} botryococcene by methylation, and that methionine was possibly the important methyl group donor, as it has been revealed that a high concentration of [Me-^{13}C] methionine was incorporated into botryococcene [16]. However, Sato et al. [15] reported that [1-^{13}C] glucose is not labelled at either position with additional methyl groups because these positions showed 0.8 to 1.5% isotopic abundance, which is the same as non-labelled, whereas C-1 and C-5 of the isoprenic units were labelled at 1.7 to 2.0% of isotopic abundance. However, many differences in the methods existed between their and our experiments. In particular, the carbon source and photo-respirative condition were mainly different from the metabolic aspect. In our experiments, the carbon source was limited to only [1-^{13}C] glucose, as compared to their experiment with CO_2 flue. The CO_2-omitted aeration we used might strongly induce photorespiration under a 12L: 12D photoperiod with a light irradiance of 100 μmol photon/m^2 s. Generally, the methyl group donor is methionine which obtains its methyl group from a one-carbon pool [19]. As the C-3 of serine and C-2 of glycine are recognized as important donors of the one-carbon pool in other organisms including humans [20] [21], the C-1 of glucose can be incorporated into both positions via the glycolytic and photorespiration pathways, respectively. The C-1 carbon on glucose selectively flows to a one-carbon pool on *B. braunii*. Obtaining more information on botryococcene methylation will provide important knowledge for further understanding the one-carbon metabolism.

4. Conclusion

In this study, 1-^{13}C-labelled glucose was clearly converted to the position predicted by the MEP pathway. We confirmed that the isoprenic precursor of botryococcene was synthesized in the Bot-22 strain via the MEP pathway. Moreover, the findings showed that additional botryococcene methyl groups were labelled, contrary to a previous report. The affect of photorespiration is one of possible factors for this phenomenon. However, more evidence is necessary to further understand methyl group metabolism in *Botryococcus*.

Acknowledgement

We sincerely thank Dr. Yoshihisa Hirakawa of the University of Tsukuba for studying the isolation and purification of microalgae. We also express our appreciation to the staff and members of our laboratory at the University of Tsukuba for their help and support. This work was financially supported by the Core Research of Evolutional Science and Technology program (CREST) from the Japan Science and Technology Agency and the Special Funds for Next Generation Energy Development Project, MEXT, Japan.

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Received 27 June 2011; accepted 2 August 2011